Identification of a Homozygous Exon-Skipping Mutation in the LAMC2 Gene in a Patient with Herlitz's Junctional Epidermolysis Bullosa

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We describe a family with the Herlitz type of junctional epidermolysis bullosa, in which the disease is associated with a homozygous splice-site mutation in the γ2-chain gene (LAMC2) of laminin-5. The mutation consists of a G-to-T substitution resulting in the out-of-frame skipping of exon 7, a frame shift, and premature stop codon accompanied by a severe reduction in the level of mRNA from the mutant allele. The distribution of the wild-type and mutated γ2-chain alleles in family members implicates the mutation in the pathology and confirms the haplotypes of the healthy carriers previously determined by genetic linkage analysis. Our results confirm that the lethal Herlitz junctional epidermolysis bullosa phenotype is caused by mutations resulting in an altered synthesis of laminin-5. ***Key words: laminin/nicein/kalinin/basement membrane/dermoepidermal junction/hemidesmosome.***
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Junctional epidermolysis bullosa (JEB) is an autosomal recessive genodermatosis characterized by marked skin fragility with the development of blisters as a result of minor trauma [1]. The pathologic hallmark of the disease is tissue separation within the lamina lucida of the epidermal basement membranes, the cleavage plane lying below the basal keratinocytes. Among the JEB variants identified clinically, Herlitz's JEB (H-JEB) represents the most severe and perhaps the most frequent form (greater than 50% of cases) [2]. H-JEB is distinguished by generalized blistering with erosions of the skin and mucous membranes, and is frequently lethal in early childhood. The non-Herlitz forms are characterized by chronic and localized blistering with no apparent shortening of the patients' life span. In JEB, disruption of the epithelial–basement-membrane interaction is often accompanied by a loss of integrity of the hemidesmosomes and anchoring filaments [1]. Recent data have suggested a role for anchoring filament proteins in the etiology of JEB because antibodies to laminin 5, which recognize their respective antigenic epitopes within the anchoring filaments, display attenuated or absent immunoreactivity with H-JEB skin ([3] and references therein).

Laminin 5, a 400-kDa noncollagenous protein that consists of three disulfide-linked subunits (α3, β3, γ2), is synthesized and secreted by basal keratinocytes of specialized squamous epithelia [4]. The cDNA sequences encoding the laminin-5 subunits are unique, and the corresponding mRNAs are products of distinct genes localized to human chromosomes 1 (β3 and γ2) [5] and 18 (α3).¶ Mutations in the gene for the γ2 subunit of laminin-5 (LAMC2) have been demonstrated recently in JEB [3,6]. Specifically, a homozygous nonsense mutation in exon 3 of the gene has been demonstrated in a family with H-JEB [3]. Furthermore, a homozygous mutation, resulting in in-frame skipping of exon 9 of the same gene, and a heterozygous insertion/deletion mutation in exon 16, resulting in a premature termination codon in one allele, also have been demonstrated in two patients with distinct forms of JEB [6].

In this study, we report the identification of a novel LAMC2 mutation in an H-JEB patient resulting in homozygous out-of-frame exon skipping, frame shift, and premature termination codon.

RESULTS AND DISCUSSION

The proband was male, born from a union of clinically unaffected first cousins. He had five brothers and sisters with H-JEB and four unaffected sisters [3]. Shortly after birth, periungual blisters on both hands and severe generalized erosions of the skin and buccal epithelia were noted. The patient died at the age of 6 weeks. Histologic examination of involved skin revealed separation of the epidermis from the underlying mesenchyme, with dermoepidermal cleavage located at the level of the lamina lucida. All the clinical, immunofluorescent, and ultrastructural characteristics were consistent with the diagnosis of H-JEB [2].

Previous analysis of skin biopsy specimens and cultured keratinocytes at the protein and mRNA level had revealed a lack of expression of the laminin γ2 chain [7], and genetic studies on the family confirmed a linkage of the clinical phenotype to the LAMC2 locus [3]. To identify the genetic defect underlying the disease, we

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Abbreviations: ASO, allele-specific oligonucleotide; H-JEB, Herlitz's junctional epidermolysis bullosa.

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first searched for gross abnormalities in the LAMC2 gene. Southern blot analysis of the H-JEB genomic DNA upon digestion with a variety of restriction enzymes did not reveal any evidence of deletions, insertions, or rearrangements in the gene. Therefore, we searched possible mutations by scanning the entire open reading frame of the cDNA (3,6 kb) reverse-transcribed from LAMC2 mRNA isolated from cultured keratinocytes of the proband. The cDNA was subjected to polymerase chain reaction (PCR) amplification using eight pairs of primers, which yielded overlapping amplimers. The size of the amplified product was verified by electrophoresis on agarose gels and compared with that of unaffected controls. In the case of the proband, the PCR product obtained with the primers flanking nucleotides (nt) 706 and 1145 revealed a band with altered electrophoretic mobility as compared to the corresponding cDNA fragment obtained with control mRNA **(Fig 1*A*)**. Specifically, a unique band of 248 bp was detected, as compared to the normal size of 439 bp, which was expected from the cDNA sequence. No evidence for the normal-size band was observed, suggesting that the patient was homozygous with respect to the 248-bp band.

Subsequent sequencing of the shortened PCR product revealed the presence of a deletion of 191 bp corresponding to nucleotides 881-1070 in the cDNA of the proband **(Fig 1*B*)**. No additional mutations were found within this amplimer or in the other PCR products corresponding to the open reading frame of the full-length cDNA. The deletion causes a frame shift in the open reading frame downstream from amino acid 254 and results in a premature stop codon (TAA) at amino acid 256, predicting a truncated laminin-γ2 polypeptide. The deletion corresponds to exon 7 of the LAMC2 gene (K. Tryggvason, unpublished results). To ascertain the muta-

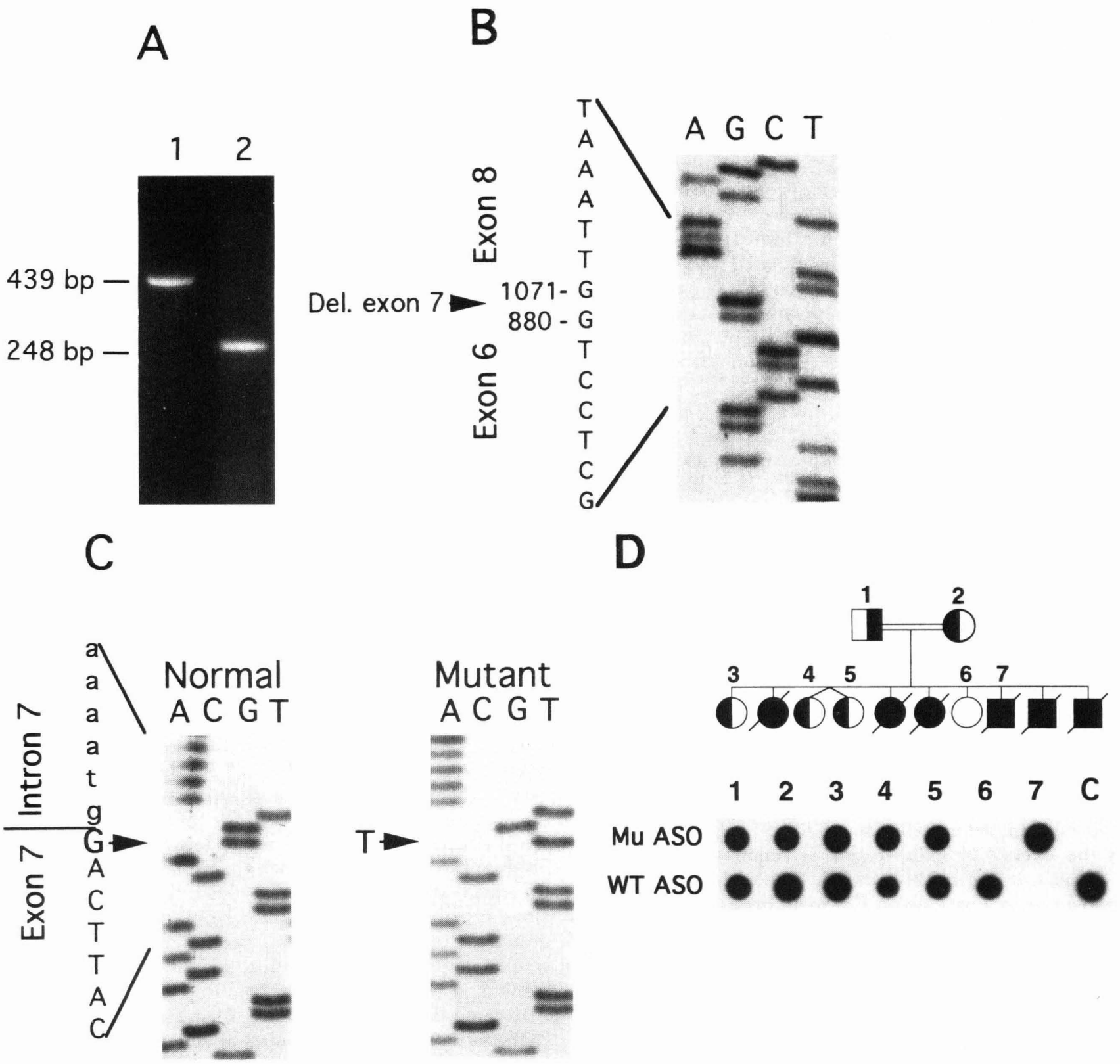

Figure 1. Identification of the point mutation in the LAMC2 gene of the H-JEB proband. (*A*) Overlapping regions of the mRNA for the γ2 chain of laminin 5 were subjected to reverse-transcription PCR and analyzed by agarose gel electrophoresis. The amplification product, extending from nt 706 to 1145 and corresponding to domain IV of the protein, demonstrated a 439-bp fragment in healthy controls (*lane 1*) and a shortened 248-bp fragment in the H-JEB patient (*lane 2*). (*B*) Nucleotide sequencing of the mutated H-JEB cDNA. By comparison with the wild-type cDNA sequence [9], a 189-bp segment corresponding to nucleotides 881 to 1070 is missing; this deletion corresponds to exon 7 of the LAMC2 gene. (*C*) Sequencing of the genomic DNA from the H-JEB patient revealed a homozygous G-to-T transversion at the 5′ donor splice site in the exon-7–intron-7 border. (*D*) Linkage of the H-JEB phenotype to inheritance of the 1070 G→T mutation using a wild-type (WT) and a mutated (Mu) ASO. The parents (*dots 1 and 2*) and three of the proband's sisters (*dots 3, 4, and 5*) are healthy carriers. A proband's sister (*dot 6*) and an unrelated healthy control (*C*) show no evidence for the presence of the mutated allele, whereas homozygosity of the proband for the mutated allele is clearly demonstrated.

tion in the genomic DNA resulting in the exon skipping, the DNA sequences corresponding to the 3′ end of exon 7 of LAMC2 were amplified by PCR using genomic DNA of the patient and healthy controls as templates, and then sequenced. A G-to-T substitution at nt 1070 was identified, which abolishes the consensus 5′ donor splice site, CAGgtaaaa [8], at the junction of exon 7 and intron 7 **(Fig 1*C*)**. The mutation, designated as 1070 G→T, results in skipping of exon 7 from the mRNA transcript, with subsequent frame shift and premature termination codon.

To verify that the homozygous G-to-T substitution is linked to the H-JEB phenotype, we confirmed the presence of the mutation on the DNA of seven members of family A. A 200-bp cDNA fragment spanning the exon-7–intron-7 junction of the LAMC2 gene was amplified by PCR using genomic DNA from each member of the family as template. The amplified products were dot-blotted on a nitrocellulose sheet and subsequently hybridized with two 20-mer allele-specific oligonucleotides (ASO) corresponding to the wild-type and mutant sequences, 1070 G→T, respectively. The PCR-amplified samples from the proband hybridized only with the mutant ASO, whereas the control and a healthy sister showed strong hybridization signal only with the wild-type ASO, indicating that occurrence of the H-JEB phenotype correlates with homozygosity for the mutant allele. The samples obtained from the parents and the remaining unaffected sisters hybridized to both the mutant and the wild-type ASO, indicating that these healthy members of the family are heterozygous carriers for the mutation **(Fig 1*D*)**. These results thus confirm that the H-JEB phenotype in this family is linked to the mutation 1070 G→T and are concordant with the segregation of the mutated LAMC2 allele demonstrated previously by the genetic analysis [3]. The possibility that the G-to-T transversion represents a polymorphic variation in the population was tested by screening PCR-amplified genomic DNA from 100 unrelated individuals. The mutation was not found in any of these samples (not shown).

We have therefore identified a novel mutation in the LAMC2 gene responsible for an H-JEB phenotype. This G-to-T transversion found in the homozygous state in the proband abolishes the 5′ donor splice site of intron 7 and leads to out-of-frame exon skipping, which affects the globular domain IV of the laminin γ2-chain precursor **(Fig 2)**. The mutated LAMC2 gene thus encodes a γ2-chain precursor composed of only domain V and truncated domain IV, which are excised during extracellular processing of the normal protein [9]. Consequently, the assembly of a functional heterotrimeric α3β3γ2 laminin-5 molecule is perturbed, presumably resulting in structural alterations of the anchoring filaments and disrupting the adhesion of basal epithelial cells to the underlying matrix. The nature of this mutation also explains the absence of the laminin γ2 chain in the dermoepidermal basement membrane of this H-JEB patient and the inability to detect the corresponding mRNA, as reported previously [7].

In higher eukaryotes, the last three nucleotides of exon sequences contribute to the recognition of the 5′ splice sites, and integrity of the overall 5′ splice region is required for correct processing of nuclear pre-mRNA [8,10]. Given that the splice junction between exon 7 and intron 7 is weak because there is no G at position +5 (gtaaaa), mutation of the G at −1 apparently renders this splice site nonfunctional. This results in an abnormal splicing process, generating a mature mRNA in which exon 6 and exon 8 are ligated together upon excision of exon 7 and the flanking introns 6 and 7. The mutation is also associated with low steady-state levels of the mRNA encoding the laminin γ2 chain [3,7].

A link has been established between the occurrence of nonsense or frame-shift mutations generating premature translational termination codons and low steady-state levels of the affected mRNAs ([11] and references therein). Such mutations are thought to interfere with the nuclear translocation and/or translation of the mutated mRNA molecules, resulting in degradation of aberrant mRNA and bearing premature termination codons.

The degree of severity of the six clinically recognized subtypes of JEB may reflect a range of functional alterations within the laminin-5 chain(s) consequent to mutations in the corresponding genes. In addition to the mutation presented in this study, we have recently linked a severe H-JEB phenotype to a homozygous mutation (R95X) that results in the degradation of mRNA encoding a truncated laminin γ2-chain precursor [3]. This mutation consisted of a C-to-T substitution, resulting in a premature stop codon in exon 3 of LAMC2. In two additional cases with different forms of JEB characterized by less severe clinical involvement, the mutation also resided in the LAMC2 gene [6]. In one case, the mutation (1184-1 G→A) results in the homozygous in-frame deletion of exon 9 of the laminin γ2-chain precursor, which impairs maturation of the protein. In the other case, a heterozygous out-of-frame deletion-insertion (2336 del20→G) in LAMC2 was predicted to result in the synthesis of one laminin γ2 chain with a truncated carboxy-terminus, whereas the mutation in the other allele remains unknown. Collectively, these results suggest that mutations in LAMC2 causing the lethal H-JEB phenotype may result in premature termination codons and altered synthesis of laminin γ2 polypeptides **(Fig 2)**.

Because the two other chains of laminin-5 have been cloned (¶, [12]), future comparative studies of additional JEB families will be helpful to establish the correlation between specific mutations in these genes and the phenotypic characteristic, thus allowing accurate pre- and postnatal diagnosis, genetic counseling, and the development of appropriate therapies.

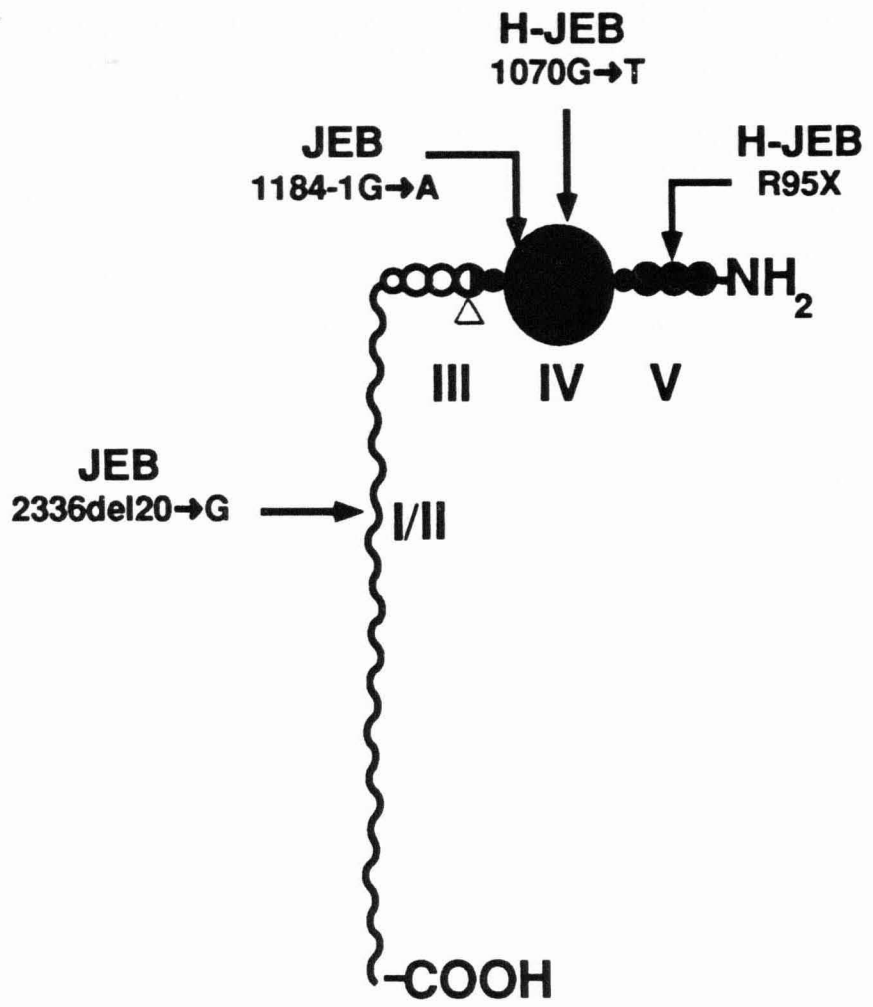

Figure 2. Schematic representation of the laminin γ2-chain precursor polypeptide. The locations of the mutation 1070 G→T and previously localized mutations [3,6] are indicated. The short arm of the laminin γ2 chain consists of epidermal growth factor–like repeats, illustrated by *small circles* (domains III and V) flanking globular domain IV. The long arm (domain I/II) is depicted by a *wavy line*. The *open arrowhead* shows the site of proteolytic processing of the precursor polypeptide; the cleaved N-terminus of the polypeptide is shown in black.

MATERIALS AND METHODS

Samples DNA was extracted from peripheral blood or from primary cultures of dermal fibroblasts [13], and total RNA was purified from keratinocytes cultured on a feeder layer of irradiated mouse 3T3 cells (ICN), as described previously [7].

Reverse-Transcription PCR Fifty micrograms of total RNA from the JEB patient and unaffected controls was reverse-transcribed in a volume of 100 μl using MMLV reverse transcriptase (Gibco-BRL). One microliter of the reaction product was used to amplify overlapping regions of the cDNA that spanned the open reading frame. The primer pair used to identify the mutation 1070 G→T (see *Results*) was (L): 5′CGCAGCTCTGCAGAATACAG3′ and (R): 5′AGATTCCGCAGTAACCTTCG3′, which amplifies the nucleotide segment from 706 to 1145 of the laminin γ2-chain cDNA sequence [9]. PCR cycling conditions were 94°C for 5 min, followed by 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 45 seconds (35 cycles), and extension was at 72°C for 5 min. Aliquots of 5 μl were run on 2% agarose gels. Amplified products were visualized under ultraviolet light in the presence of ethidium bromide and photographed. Amplified cDNA fragments with altered mobility were subcloned into a TA vector (Invitrogen) and sequenced using the dideoxy termination method [13].

Verification of the Mutation in the H-JEB Kindred PCR reactions on genomic DNA (50 ng) were carried out using primers (R): 5′CATGATGTGATTCTGGAAGG3′ and (L): 5′CTTCAATCATAGTCTGCTCT3′, which are located in exon 7 and intron 7 of the LAMC2 gene, respectively (K. Tryggvason, unpublished results). The cycling conditions for PCR were 95°C for 7 min, followed by 94°C for 45 seconds, 53°C for 45 seconds, and 72°C for 45 seconds (35 cycles), and extension was at 72°C for 5 min. Twenty microliters of the resulting 200-bp product of the PCR reaction was transferred onto a Zeta-probe membrane (BioRad) using a dot-blot apparatus. The membranes were prehybridized in a solution of 5 × SSPE, 0.5% sodium dodecylsulfate, and 5 × Denhart's for 30 min at 37°C, and then hybridized for 1 h at 37°C with either the ^{32}P-labeled wild-type ASO (5 × 10^6 cpm/ml) with the sequence 5′CAAGACTTACACATTCAGGT3′ or the mutated ASO 5′CAAGACTTACACATTCATGT3′. Filters were washed in 2 × SSPE, 0.1% sodium dodecylsulfate for 15 min at room temperature and 15 min at the T_m of the oligonucleotides, and exposed for autoradiography.

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